

Viroids are single-stranded covalently closed circular RNA molecules existing as highly base-paired rod-like structures

(electron microscopy/endgroup analysis/ultracentrifugation/thermal denaturation)

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ABSTRACT Viroids are uncoated infectious RNA molecules pathogenic to certain higher plants. Four different highly purified viroids were studied. By ultracentrifugation, thermal denaturation, electron microscopy, and end group analysis the following features were established: (i) the molecular weight of cucumber pale fruit viroid from tomato is 110,000, of citrus exocortis viroid from *Gynura* 119,000, of citrus exocortis viroid from tomato 119,000, and of potato spindle tuber viroid from tomato 127,000. (ii) Viroids are single-stranded molecules. (iii) Viroids exhibit high thermal stability, cooperativity, and self-complementarity resulting in a rod-like native structure. (iv) Viroids are covalently closed circular RNA molecules.

Viroids exert biological functions like viruses since they are infectious and pathogenic. In contrast to viruses, however, all known viroids seem to exist only as uncoated RNA molecules and their genetic information is not sufficient to code for a protein with a molecular weight larger than 10,000. They are pathogens of certain higher plants. Little is understood, as yet, about their mechanisms of replication and pathogenicity. Moreover, the available information is too limited to lead to a concise structural model of viroids (1). Previous data on molecular weights of viroids were obtained by indirect methods and the determinations were based on particular assumptions about their unknown conformation. Analysis in sucrose gradients, in polyacrylamide gels, and in the electron microscope resulted in molecular weight values ranging between 25,000 and 150,000 for both the viroid of the spindle tuber disease of potato (PSTV) (e.g. refs. 2-4) and the viroid of the exocortis disease of citrus (CEV) (e.g. refs. 5-7). The sedimentation coefficients reported range from 4 S to 25 S (2, 6, 7). A considerable number of structural models have been proposed (e.g. refs. 4, 7, and 8); single strands with hairpins or double strands with incomplete base pairing have been considered as the most probable structure of viroids.

The availability of highly purified viroid RNA in sufficient quantities enabled us to investigate the structure in detail.

MATERIALS AND METHODS

Materials. The source of viroids, their propagation in the host plants tomato (variety Rentita) and *Gynura aurantiaca* DC, and the bioassay were as described[†]. The enzymes and buffers used are specified under *Results*.

Purification of Viroid RNA. Total nucleic acids were extracted routinely from batches of 2 kg of viroid-infected plant

tissue with the aid of phenol-sodium dodecyl sulfate and 0.1 M Tris-HCl (pH 8.0) and fractionated with 2 M NaCl and cetyltrimethylammoniumbromide as described[†]. The fraction containing the viroid RNA was subjected to preparative gel electrophoresis, and the stained viroid band (Fig. 1) was cut out and stored frozen. Viroid RNA was extracted from the gel by homogenization in 0.1 M Tris-HCl (pH 7.5), 0.1 M NaCl, and 0.1 mM EDTA in the presence of 20% (vol/vol) phenol, adsorbed on DEAE-cellulose, desorbed with 1.2 M NaCl, and precipitated with ethanol. The electrophoretic separation and purification was repeated three times.

Gel Electrophoresis. Preparative slab gels (250 × 230 mm, 6 mm thick) were run in Tris-borate-EDTA, pH 8.3 (9) in the presence of 8 M urea at 350 V, 60 mA for 20 hr. Gels contained 5 or 10% acrylamide and 0.125 or 0.25% bisacrylamide.

Determination of Molecular Weight and Sedimentation Coefficient. All experiments were done in a Spinco model E ultracentrifuge, equipped with a photoelectric scanner and an electronic multiplexer. The baseline in the equilibrium runs was determined as described (10). The concentration profiles were evaluated from the formula for monodisperse solutions:

$$\Delta \ln c = [M(1 - \bar{v} \cdot \rho) \omega^2 / 2 RT] \cdot \Delta r^2,$$

where M is the molecular weight, \bar{v} the specific volume, ρ the density, and $\omega/2\pi$ the number of revolutions per sec.

Melting Curves. Differential melting curves were determined as described (11).

Electron Microscopy. Viroid RNA was prepared for electron microscopy by the formamide-urea modification of basic monolayer techniques (12), with benzyltrimethylammonium chloride (13) as film forming surfactant in the spreading solution. Thin carbon support films and high-contrast staining with ethanolic uranyl salts (14) were used to contrast double- and single-stranded RNA efficiently. Details of preparation steps are described in the figure legends.

The Elmiskop 102 is operated in darkfield by hollow-cone illumination at 100 kV and operational magnifications of 50,000 and 100,000 times.

End Group Analysis. 5'-Labeling of RNA from the viroid of pale fruit disease of cucumber (CPFV) was attempted using the phosphate exchange catalyzed by bacteriophage T4-induced polynucleotide kinase (15). [γ -³²P]ATP (specific activity 50 Ci/mmol) was synthesized according to Glynn and Chappell (16). Incubation volume of 20 μ l contained: CPFV RNA, 0.25 A₂₆₀ unit (about 100 pmol); [γ -³²P]ATP, 800 pmol; ADP, 800 pmol; Tris-HCl, pH 7.5, 2 μ mol; MgCl₂, 0.2 μ mol; mercaptoethanol, 0.3 μ mol; and bacteriophage T4-induced 5' polynucleotide kinase, 12 units. (One A₂₆₀ unit is that amount of material that gives an absorbance of 1 when dissolved in 1 ml of solvent when the light path is 1 cm.)

Abbreviations: PSTV, viroid of the spindle tuber disease of potato; CEV, viroid of the exocortis disease of citrus; CPFV, viroid of the pale fruit disease of cucumber; A₂₆₀ unit, that amount of material that gives an absorbance of 1 when dissolved in 1 ml of solvent when the light path is 1 cm.

[†] H. L. Sängner, and D. Riesner, submitted to *J. Gen. Virol.*

Table 1. Sedimentation coefficients and molecular weights of viroids

Viroid	Host plant	Sedimentation coefficient $s_{20,w}^0$ (S)	Molecular weight	No. of calculated nucleotides*
Citrus exocortis	<i>Gynura</i>	6.7 ± 0.1	$119,000 \pm 4,000$	357
Citrus exocortis	Tomato	6.7 ± 0.1	$119,000 \pm 4,000$	357
Potato spindle tuber	Tomato	6.7 ± 0.1	$127,000 \pm 4,000$	381
Cucumber pale fruit	Tomato	6.5 ± 0.1	$110,000 \pm 5,000$	330

* Based on an average molecular weight of 322 per nucleotide plus an average weight value of 11 per nucleotide from bound Mg^{++} and Na^+ ions according to the data of Krakauer (18).

After 60 min at 37° , the incubation mixture was fractionated by electrophoresis on a 10% polyacrylamide slab gel in the presence of 7 M urea and analyzed by autoradiography and subsequent staining of the gel.

3'-Labeling of CPFV RNA was attempted exactly as described for tRNA (17): 0.4 A_{260} unit of CPFV RNA (about 160 pmol) were treated with excess sodium metaperiodate followed by reduction with excess potassium $[^3H]$ borohydride (specific activity 3.3 Ci/mmol). The mixture was dialyzed against distilled water at 4° . tRNA (500 μg) was added as carrier, and the RNA was precipitated with ethanol and collected by centrifugation. The pellet was dissolved in 20 μl of water and analyzed by electrophoresis on a 10% polyacrylamide gel. The gel was stained and cut in 3 mm-slices. The slices were solubilized with ammonia/ H_2O_2 and radioactivity was determined in Aquasol 2 (New England Nuclear).

RESULTS

Homogeneity and Infectivity of Viroids. The four viroids investigated are listed in Table 1. In the RNA fractions from viroid-infected plant tissue soluble in 2 M NaCl, an extra RNA band is found in preparative 5% polyacrylamide slab gels (Fig. 1a) as compared to the corresponding sample from healthy plants (Fig. 1b). This extra RNA band was identified as viroid by its infectivity. After the final purification the viroid RNA was electrophoretically homogeneous on 5, 10 (Fig. 1c), and

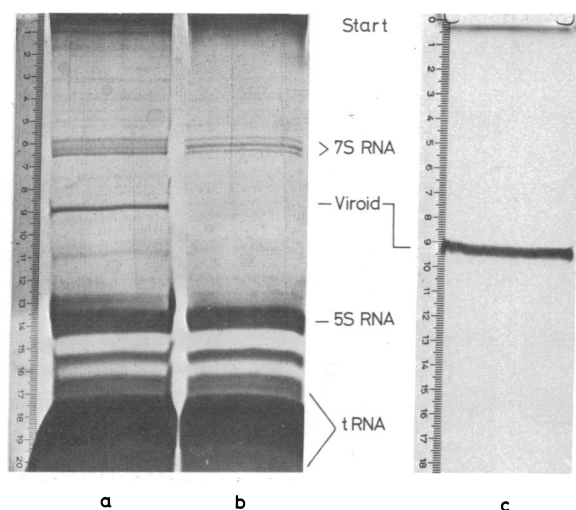


FIG. 1. Preparative separation of nucleic acids from healthy and from viroid-infected tomato leaves by electrophoresis on polyacrylamide slab gels in the presence of 8 M urea after staining with methylene blue. (a) Profile of 30 mg of the RNA fraction soluble in 2 M NaCl from viroid (CPFV)-infected tomato leaves on a 5% gel (6 mm thick). (b) Profile of 30 mg of the corresponding fraction from healthy tomato leaves on the same gel. (c) Band of 200 μg of purified viroid (CPFV) RNA in the final 10% gel (3 mm thick) after two preceding electrophoretic separations on 5% gels.

15% polyacrylamide gels; impurities were below 0.5%. In dilution-end-point titration on a molar basis with highly purified samples we repeatedly observed that about 10% of the tomato plants became infected when they were inoculated with a solution containing 50 to 100 molecules per plant. In view of the relative insensitivity of the plant bioassay, this result indicates an unusually high specific infectivity and stability for the purified viroids investigated in this study.

Molecular Weights and Sedimentation Coefficients of Native Viroids. Native viroids were studied in 0.01 M sodium cacodylate (pH 6.8), 0.1 M NaCl, 5 mM $MgCl_2$, and 1 mM EDTA. In velocity sedimentation experiments all viroids showed a single homogeneous boundary. The sedimentation coefficients listed in Table 1 are in the narrow range of 6.5–6.7 S. They are identical when determined at 10° or 22° . The molecular weights were determined at 10° by high-speed (15,000 rpm) as well as low-speed (7000–8000 rpm) equilibrium experiments. Initial concentrations were between 0.2 and 0.4 A_{260} unit/ml. The concentration profiles, when plotted according to $\Delta \ln c$ against Δr^2 and evaluated over a concentration range of more than an order of magnitude, gave straight lines with high accuracy. The molecular weights are listed in Table 1. They are based on a partial specific volume $\bar{v} = 0.53 \text{ cm}^3/\text{g}$, which is the known value for the sodium salt of a crude mixture of tRNA from yeast (19). The same value can be safely used for viroid RNA since (i) the degree of base-pairing and the composition of nucleotides are comparable in CEV and tRNA (8), (ii) the particular tertiary structure of tRNA has only minor influence on \bar{v} (19), and (iii) very similar values of \bar{v} have been determined for the sodium salt of 16S ribosomal RNA from *Escherichia coli* (20) and for RNA from brome mosaic virus (21). The molecular weights of the four viroids are in a narrow range between 110,000 and 127,000. This corresponds to a chain length of about 330 and 380 nucleotides, respectively, and indicates a significant difference between the smallest (CPFV) and the largest (PSTV) viroid studied.

Thermal Denaturation. Denaturation profiles were determined by UV melting curves; buffer conditions of low ionic strength (0.01 M sodium cacodylate, pH 6.8, 1 mM EDTA) had to be chosen to obtain melting temperatures below 60° . In Fig. 2 a differential melting curve of CPFV is shown which is representative for all viroids tested. The particular features of the melting process of viroids are displayed best by comparison with a melting curve of tRNA obtained under identical conditions. From Fig. 2 and from quantitative thermodynamic and kinetic analysis (K. Henco, D. Riesner, and H. L. Sanger, in preparation) it becomes evident that viroid RNA is characterized by high thermal stability, high cooperativity, and a high degree of base-pairing. From the wave-length dependence of the hypochromicity, the G+C content of the base-paired segments was estimated as 70%. The observed hypochromicity of 22% indicates a degree of refolding of the nucleotide chain comparable to that of tRNA, 5S RNA, or 6S RNA (e.g., ref. 22), as

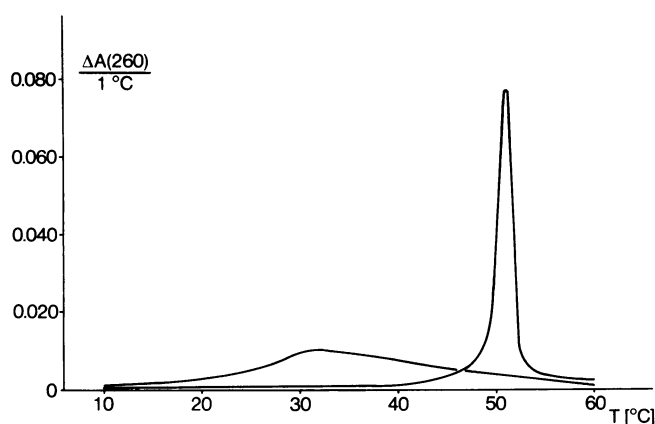


FIG. 2. Differentiated melting curves of CPFV RNA (narrow curve) and tRNA^{Phe} from yeast (broad curve) in 0.01 M sodium cacodylate (pH 6.8), 1 mM EDTA. The values of $\Delta A(260 \text{ nm})/^\circ\text{C}$ refer to a RNA concentration of 1 A_{260} , 1 cm optical path length, and 20°.

has been suggested for CEV RNA (8). From the width of the melting curve it is estimated that more than 60 base-pairs melt in a single all-or-none transition. Therefore, the complementary sequences of viroid RNA must form at least one long and fairly regular double helix and not only short hairpins like tRNA, 5S RNA, and 6S RNA (22).

Molecular Weights of Denatured Viroids. In order to decide whether native viroid RNA is composed of two recombined strands or consists of a single nucleotide strand that may form base-paired regions because of self-complementary sequences, the molecular weights of fully denatured viroids were studied. Sedimentation equilibrium experiments were done in 0.01 M sodium cacodylate (pH 6.8), 0.05 M NaCl, 1 mM EDTA, and 8 M urea at 25°. Under these buffer conditions the midpoint temperature of denaturation of PSTV is 22.6°; at 25° the denaturation is more than 95% complete. The partial specific volume \bar{v} of RNA in 8 M urea was taken from a reference experiment of tRNA under the same buffer conditions. The molecular weight of fully denatured tRNA in 8 M urea is the same as that of native tRNA in the absence of urea and the change in $M(1 - \rho\bar{v})$ is due to the changes in ρ and \bar{v} . A value of $\bar{v} =$

0.58 ml/g was obtained, from which the molecular weight of denatured viroid was determined to be $112,000 \pm 10,000$. Within the limits of error the molecular weights of the native and denatured viroids are the same. Therefore, one has to conclude that no strand separation occurs during denaturation.

Hydrodynamic Shape of Native Viroids. From the frictional ratio f/f_0 one may derive information about the shape of viroids in solution. A value of $f/f_0 = 2.5$ is calculated from the sedimentation coefficient, molecular weight, and partial specific volume. Since viroid RNA is highly base-paired, one may assume a fairly rigid structure, and apply as a first approximation the hydrodynamic theory of rotational ellipsoids. In that case f/f_0 is a rough measure of asymmetry and/or hydration (23). Since the hydration of viroid RNA is presumably similar to that of other RNAs, a ratio of long axis to short axis of a prolate ellipsoid of about 20 is obtained for the viroids, which indicates a highly extended structure. For comparison, the f/f_0 value of tRNA is 1.4, whereas a piece of double-stranded DNA with about the same molecular weight as viroid RNA (24) has a frictional ratio of 2.9 and an axial ratio of about 25. From these comparisons it is evident that viroids have much more similarities to a short and rod-like piece of native DNA than to the more globular tRNA.

Electron Microscopy of Native Viroid RNA. Fig. 3 shows an electron micrograph of native CPFV RNA. Within the uniform population of molecules no contaminating nucleic acid strands of cellular origin are detectable, reflecting the purity of the viroid preparation examined. The shape of the molecules can be described by rods or dumb-bells. In view of the molecular weight of 110,000 for CPFV RNA, corresponding to 330 nucleotides, the measured average particle length of 350 Å implies a high degree of base-pairing and condensation by coiling. The value of about 20 for the ratio of long axis to short axis from our hydrodynamic data cannot be expected to agree quantitatively with the electron microscopic data since the diameter is affected by the stain and the image processing. Rod-like structures have been reported for PSTV (4). In our electron micrographs (Fig. 3, CPFV) most of the RNA molecules are dumb-bells that are asymmetric with respect to the size of their terminal knots. The same result was found for all the four viroid RNAs investigated.

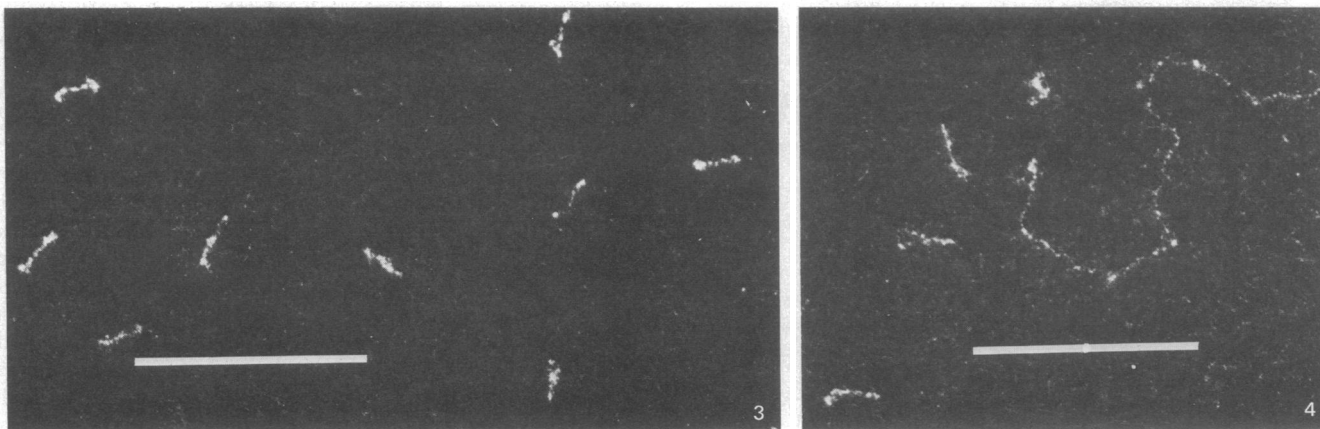


FIG. 3. Electron micrograph of native CPFV RNA. Viroid RNA (1.5 $\mu\text{g/ml}$) was spread in 0.5 M ammonium acetate containing 25 $\mu\text{g/ml}$ of benzyldimethylalkylammonium chloride without any denaturation steps included; 50 μl were spread on 70 cm^2 water surface. Scale line represents 1000 Å.

FIG. 4. Comparison of CPFV RNA and ribosomal RNA from *Acholeplasma laidlawii* under denaturation conditions. The RNA solution contained both nucleic acids in equal amounts (1.5 $\mu\text{g/ml}$ of final concentration). Denaturation was performed by heat-treatment (5 min at 60°) in the presence of formamide (80%), urea (4.8 M), and formaldehyde (0.4%). Benzyldimethylalkylammonium chloride (25 $\mu\text{g/ml}$) was added and samples were spread on water. Scale line represents 1000 Å.

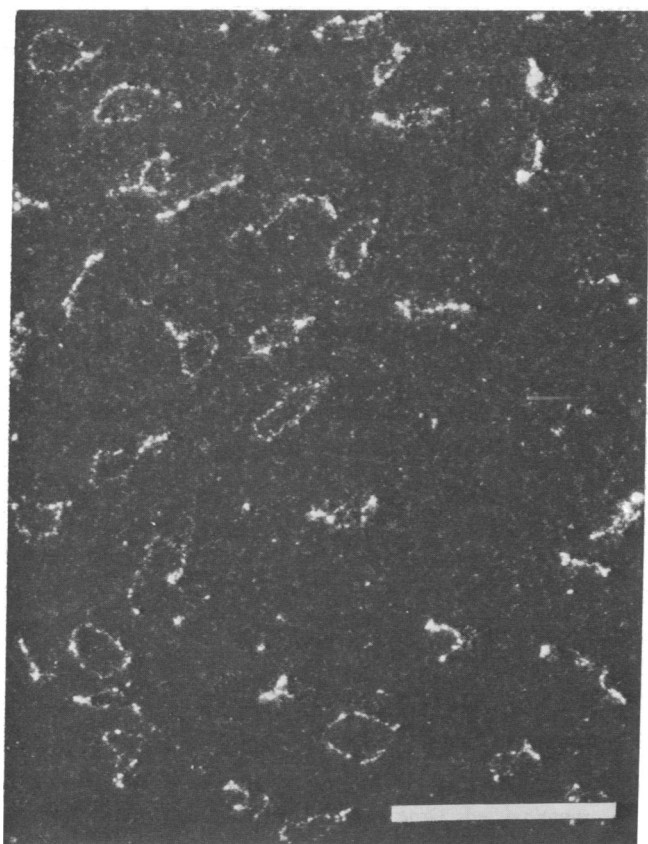


FIG. 5. Electron micrograph of denatured viroid RNA. Representative population of CPFV RNA molecules showing various types of structures ranging from rods and dumb-bells (see Fig. 3) to single-stranded circles. The intermediate structures like tennis rackets and balloons represent different states of renaturation. Nicked molecules (0.5–1.0%), which do not fit into these categories, are usually found in our preparations. Denaturation and spreading was as described in the legend of Fig. 4. Scale line represents 1000 Å.

Electron Microscopy of Denatured Viroid RNA. Figs. 4 and 5 represent viroid RNA molecules (CPFV) mounted on support films after treatment under strongly denaturing conditions (see figure legends). In Fig. 4, ribosomal RNA of mycoplasma (*A. laidlawii*) was added in order to prove the successful unfolding of the secondary structure of single-stranded nucleic acids. The

ribosomal RNA molecule appears fully extended and thinner than the condensed segments of viroids. In contrast to the uniform native material, a variety of structures is found ranging from rod-like molecules to fully denatured single-stranded circles. Typical intermediate forms with contours like tennis rackets are clearly recognizable in Fig. 5 and represent the majority of the population. Balloon-like structures with a rather short base-paired region are also found. Nicked molecules (0.5–1.0%) are usually present in our preparations. Their number could be significantly increased by controlled RNase treatment. All these asymmetric structures substantiate the existence of regions rich in G+C, as detected in our analysis of the melting behavior of viroid RNA. The occurrence of the various structures in the same micrograph (Fig. 5) is explained by partial renaturation during spreading on water. It is obvious that different local environmental conditions develop during spreading and mounting. The same variety of denatured RNA structures was also obtained when viroid preparations were melted at 100° for 2 min in 1.5 mM Na₃-citrate, 15 mM NaCl (pH 7.0) in the absence of the denaturing formamide-urea mixture. In these experiments formaldehyde was added after melting to give a final concentration of 0.4%.

Incubation of the viroids with proteinase K or Pronase to digest any residual protein components possibly associated with the RNA did not alter the structures observed in the electron microscope. Identical results in all respects were obtained when PSTV RNA and the two CEV RNAs were subjected to the same analysis.

Analysis of the 5'-end. Analysis in a 10% polyacrylamide gel shows (Fig. 6) that viroid RNA is not phosphorylated at all by 5'-polynucleotide kinase. Radioactivity is only found at a position corresponding to RNA material that is moving significantly more slowly than viroid RNA and which is sometimes present in trace amount (<0.5%) in viroid preparations. It could be either nicked viroid RNA or a contaminating host RNA species. [γ -³²P]ATP plus traces of degraded material are visible in the autoradiography near the tracking dye. The efficient phosphorylation of this contaminating RNA strongly suggests the absence of an accessible 5'-end in viroid RNA.

Analysis of the 3'-end. Fig. 7 shows that CPFV RNA is not ³H-labeled upon metaperiodate oxidation and [³H]borohydride reduction. The radioactivity is again from traces of degraded material present in the viroid preparation. This result suggests that viroid RNA lacks a free 3'-end, and it also excludes the possible existence of a capped 5'-terminus containing exposed

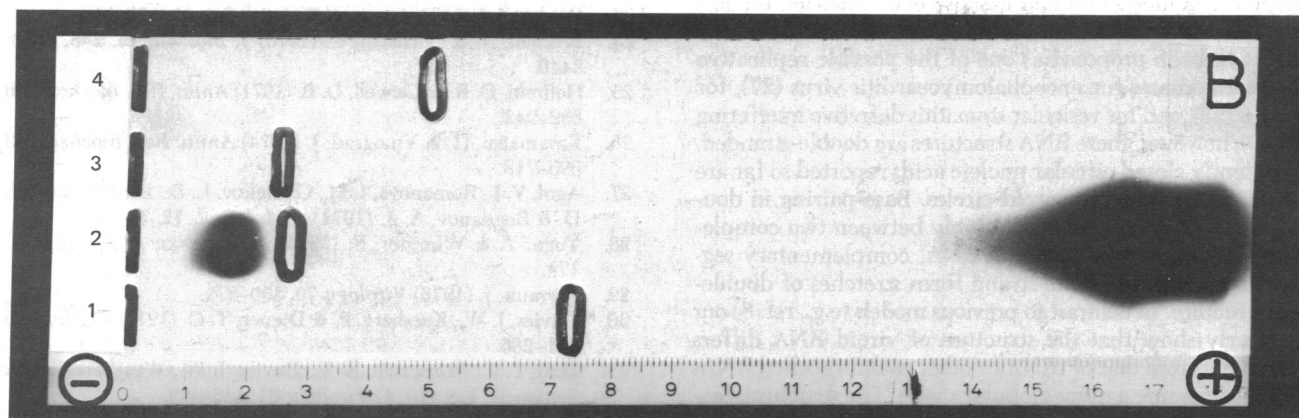


FIG. 6. Analysis on a 10% polyacrylamide slab gel of an attempted [5'-³²P]phosphorylation of CPFV RNA. RNA positions were determined by autoradiography and subsequent staining. The stained RNA bands are encircled: (1) rabbit liver tRNA^{Val}; (2) CPFV RNA after attempted [5'-³²P]phosphorylation; (3) untreated CPFV RNA; (4) 5S RNA. B, bromophenol blue tracking dye. A contaminating RNA (less than 0.5% of the viroid RNA) is efficiently phosphorylated (slot 2) and serves as an internal control for successful phosphorylation. Viroid RNA, however, which is present in a 200-fold concentration, is completely resistant to 5'-phosphorylation.

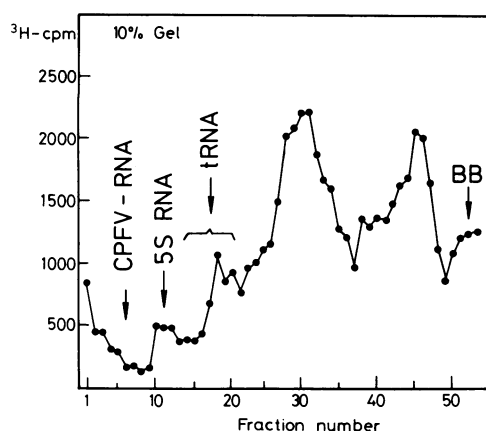


FIG. 7. Analysis on a 10% polyacrylamide slab gel of metaperiodate/[^3H]borohydride-treated CPFV RNA. Arrows indicate the positions of CPFV RNA, 5S RNA, tRNA (made visible by staining in the neighboring slots), and bromphenol blue (BB) tracking dye. In control experiments with equimolar amounts of tRNA, more than 100,000 ^3H cpm per gel slice were obtained in the peak region of tRNA.

2',3'-hydroxyls, the latter of which would have been labeled under these conditions.

DISCUSSION

Our investigation strongly suggests that viroids are single-stranded, covalently closed RNA molecules with a high degree of self-complementarity and base-pairing. This unusual structural feature, as revealed by the electron microscope, is substantiated by the data obtained from hydrodynamic and thermodynamic studies in solution. Moreover, the nature of viroids as covalently closed circular RNA molecules is supported by the biochemical evidence. Our attempts to phosphorylate viroid RNA at the 5'-terminus failed completely, and a mRNA-like structure with two 3'-terminal ribose moieties may be excluded due to the resistance of viroid RNA to metaperiodate oxidation and [^3H]borohydride reduction. The absence of a 5'-terminal triphosphate in the ^{32}P -labeled viroid RNA *in vivo* and the resistance of viroid RNA to snake venom phosphodiesterase degradation (both unpublished results) corroborate the evidence for a true RNA circle.

We have thus found single-stranded covalently closed circular RNA molecules in nature. So far, circularity is well known for double-stranded DNA and has also been reported for single-stranded DNA (25, 26). Furthermore, covalently closed circles have been proposed as one of the possible replicative RNA intermediates for encephalomyocarditis virus (27), for poliovirus (28), and for vesicular stomatitis defective interfering virus (29); however, these RNA structures are double-stranded. All covalently closed circular nucleic acids reported so far are much larger in size than viroid circles. Base-pairing in double-stranded circles occurs exclusively between two complementary strands, while in viroid RNA, complementary segments in one and the same strand form stretches of double-helical structure. In contrast to previous models (e.g., ref. 8) our data clearly show that the structure of viroid RNA differs considerably from that of tRNA or other multibranched RNAs which may assume a more globular shape. The structural fea-

tures of viroids offer an explanation for the many discrepancies in previous reports on the molecular weight as determined by indirect methods, and for the finding that viroid RNA is neither aminoacylated nor translated *in vitro* (30, 31).

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